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14. ABSTRACT

Metastasis and tumor progression at metastatic sites ultimately results in the demise of prostate cancer (PCa) patients. Currently there are no highly effective methods that can target these problems. Aptamers, which have proven clinical efficacy for non-neoplastic disease and are generally more specific and stable than antibodies, may have clinical utility in PCa. However, defining aptamers that can prevent metastasis is challenging due to the fact that many proteins that play a role in the metastatic process are unknown. The overall goal of this project is to develop novel method to inhibit cancer metastasis. The major hypothesis to be tested is that aptamers (short oligonucleotides) can be developed that target the process of invasion, without prior knowledge of a target protein, and that these aptamers will inhibit the development of metastasis. We also hypothesize that the aptamers can be used to identify cell surface proteins that are important mediators of metastasis. This latter information is important as it may help identify further therapeutic targets. We have made some initial progress towards testing this hypothesis. Specifically, we have identified aptamers, using a novel application of a process called "systemic evolution of ligands by exponential enrichment" (SELEX) {reviewed in \Brody, 2000 #10680}, that bind PCa cells that we selected for their high metastatic ability. We now propose to test these aptamers for their ability to inhibit metastasis and identify their target protein.

Introduction

Metastasis and tumor progression at metastatic sites ultimately results in the demise of prostate cancer (PCa) patients. Currently there are no highly effective methods that can target these problems. Aptamers, which have proven clinical efficacy for non-neoplastic disease and are generally more specific and stable than antibodies, may have clinical utility in PCa. However, defining aptamers that can prevent metastasis is challenging due to the fact that many proteins that play a role in the metastatic process are unknown. The overall goal of this project is to develop novel method to inhibit cancer metastasis. The major hypothesis to be tested is that aptamers (short oligonucleotides) can be developed that target the process of invasion, without prior knowledge of a target protein, and that these aptamers will inhibit the development of metastasis. We also hypothesize that the aptamers can be used to identify cell surface proteins that are important mediators of metastasis. This latter information is important as it may help identify further therapeutic targets. We have made some initial progress towards testing this hypothesis. Specifically, we have identified aptamers, using a novel application of a process called "systemic evolution of ligands by exponential enrichment" (SELEX) {reviewed in \Brody, 2000 #10680}, that bind PCa cells that we selected for their high metastatic ability. We now propose to test these aptamers for their ability to inhibit metastasis and identify their target protein.

Body

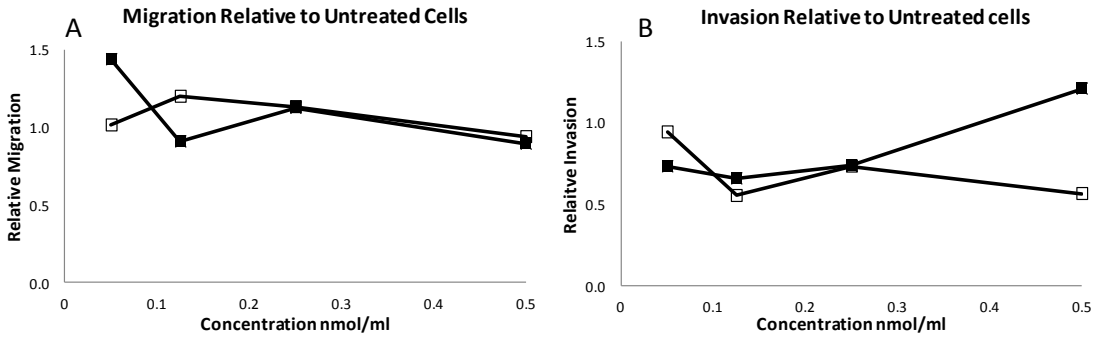
Task 1 Completion of analysis of RNA aptamers

Previous work had focused on RNA SELEX, including *invivo* and *invitro* experiments demonstrating an inhibitory effect on both invasion through a matrigel membrane and metastasis when injected into a mouse model. Work had been ongoing to identify the most suitable concentration to use and to determine other cell lines that were affected by the aptamer, but had previously been inconclusive

Task 1.1 Effect of different levels of aptamers on invasion and migration

We compared the effect of the Mod13 aptamer on PC-3luc cells to determine the optimum level of aptamers to use. No significant inhibition of invasion or migration was observed compared to scrambled RNA.

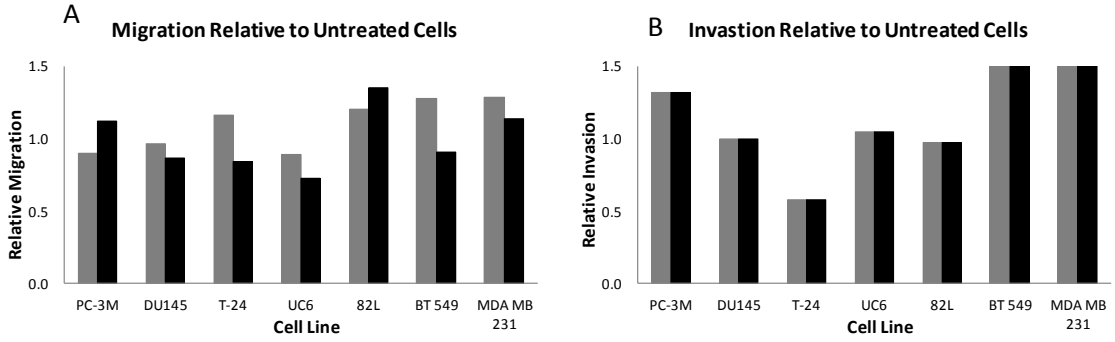
Figure 1 Invasion and migration relative to control cells. 0.5ml of cells at $2x10^5ml^{-1}$ were incubated overnight in a chamber with either a PET membrane or a matrigel layer over a PET membrane to determine invasion and migration. A) Relative Migration. B) Relative Invasion □Scrambled control; ■ AIA1.



Task 1.2 Effect of Mod13 Aptamer on Other Cell Lines

Previous work had suggested that the ability to inhibit invasion was limited to prostate cell lines. This was extended to include further cell lines. No effect on these cell lines was observed (Fig. 2).

Figure 2. Invasion and Migration Relative to Control Cells of Different Cell Lines 0.5ml of cells at $2x10^5ml^{-1}$ were incubated overnight in a chamber with either a PET membrane or a matrigel layer over a PET membrane to determine invasion and migration. A) Relative Migration. No significant inhibition of migration through a PET membrane was observed compared to scrambled RNA. B) Relative Invasion. No significant inhibition of invasion was observed. ■ Scrambled control, ■ AIA1 aptamer.



Task 1.3 Determination of binding efficiency.

Previous work had shown binding of the aptamers to the cell lines, but no quantitative analysis had been performed. To determine binding , aptamers, scrambled control and the variable region of each were labeled with Cy5 and binding to different cell lines investigated.

The results showed that although the aptamers sequence had higher binding than the scrambled RNA, there was no difference in its ability to bind the high and low invasive PC-3 luc lines and that it did not discriminate between other cancer cell lines. The variable region alone showed no difference in binding of the Mod13 region and the scrambled region (data not shown).

Task 2 Development of New Aptamer

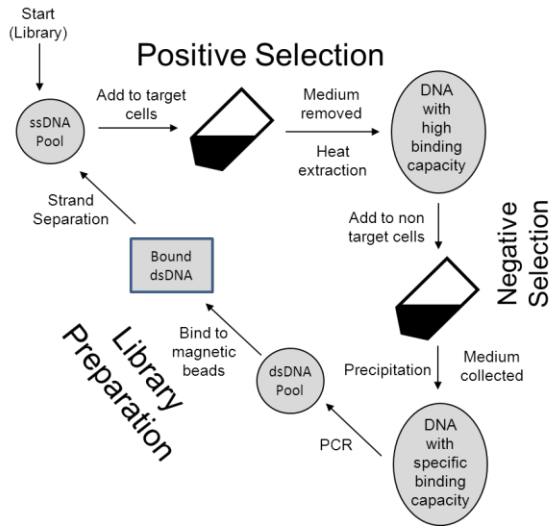
In light of our inconsistent results with our RNA-based aptamers and the reviewer's recommendations to perform additional rounds of selection, we have opted to develop a new set of aptamers for testing.

The SELEX procedure can used to create either DNA or RNA aptamers and both have been successfully used in the past, with their own advantages and disadvantages. Due to the relative stability of DNA aptamers, which would more likely translate into a clinical therapy, we elected to pursue creating new anti-invasive aptamers using selection of DNA through the SELEX procedure. The literature also supports the use of DNA molecules to bind specific cells or proteins, including selecting on cancer cells with non tumorgenic cells as the negative (e.g Ferreira 2006, Shangguan 2007, Kunii 2011, Review Phillips 2008)

Task 2.1 Development of a SELEX protocol

As there is no need for a transcription step in DNA SELEX, a modified SELEX procedure was designed

Figure 3 SELEX Procedure. An initial ssDNA library is mixed with target cells and incubated on ice for 45 minutes. The positive selection cells are washed to remove any DNA that is not bound to a target and then heated at 95C for 10 minutes to elute the DNA. This is then added to the negative selection cells and incubated again. The cells are washed to remove the non bound DNA, which is precipitated and used as the template for PCR, using a biotinylated primer. This is bound to magnetic beads and the non biotinylated strand recovered.



Throughout the SELEX procedure, approximately 100pmol ssDNA was used as the template. $1x10^6$ PC-3^{luc} high invasive cells, previously developed were used as positive selection unless otherwise stated and removed from plate without trypsin to leave cell surface protein molecules in as close to their native state as possible and to maximize the number of available protein sites for the aptamer to bind. Each stage apart from round 1, the aptamers bound to the positive selection eluted in 50µl and 25µl of this used for the negative selection.

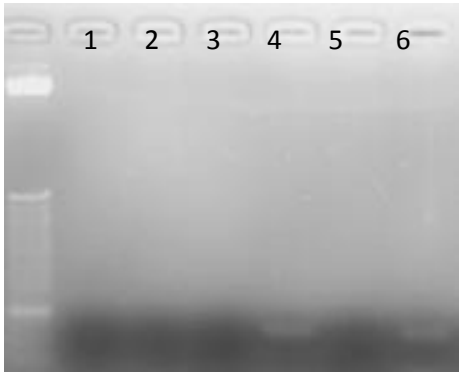
Previous studies have suggested that twenty rounds of SELEX may be necessary to isolate DNA sequences with high and specific binding affinity so a SELEX scheme was designed with the intent of performing at least fifteen rounds. This would increase the strength of the negative selection gradually, starting with a non cancerous prostate line, which was used to select against general prostate cell

surface features that are not involved in the cancer phenotype. Secondly, rounds of SELEX would be performed with a prostate cancer line that has a poor metastatic potential in order that features associated with cancer but not metastasis would be selected against. By limiting the number of rounds using these cell lines, it was anticipated that the selection would not be complete, but that no DNA molecules that bound to cell surface features that were important in invasion or metastasis would be lost. Finally, SELEX as performed with a line developed from the same parental source as the positive selection that differed only in its ability to invade through a matrigel membrane. This was expected to differ in only a few molecules and for these differences to be in terms of number of cell surface molecules rather than their presence or absence. By performing several rounds of SELEX before this cell was used, it was expected that any DNA that bound to such molecules would be present in high enough quantities that it would not be lost by binding to the features on the cell with low invasive potential.

In addition to changing the negative selection cell, within each set of SELEX rounds, the stringency increased, starting from a low number of trypsinized cells, which would have lost some of the cell surface features, to cells that were removed without trypsin, so that the tendency to select aptamers that might be internalized was reduced. At this stage, we have performed 18 rounds of selection as detailed below:

SELEX Round1, Positive selection only, $6x10^6$ cells (Fig. 4).

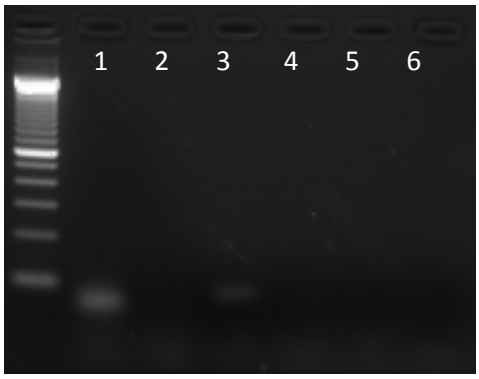
Figure 4. PCR products of SELEX Round 1. Products were seen using either the initial library as template, or the SELEX round 1 precipitated DNA. No band was observed without amplification, or with the negative control



1) No PCR
2) Negative PCR
3) Library
4) Library PCR
5) SELEX R1
6) SELEX R1 PCR

SELEX Rounds 2-18, Negative Selection. Negative selection was performed with the non cancerous prostate line BPH-1 in rounds 2-5 and with prostate cancer line LNCaP which has low invasive capabilities in rounds 6-10. In rounds 11-18, negative selection was performed with the PC-3^{Luc} low invasive line developed previously.

Figure 5, SELEX R17 PCR products. Bands were observed with the positive control, using the initial library as template and with the R17 precipitated DNA. No bands were seen with negative control or non-amplified DNA. The SELEX R17 product was a discrete single band.



1) SELEX PCR
2) SELEX no PCR
3) Library PCR
4) Library no PCR
5) H_2O control PCR
6) H_2O control no PCR

Task 2.2 Analysis of sequences developed through SELEX procedure

In order to determine the success of the SELEX procedure and if further rounds were likely to be required, sequencing was performed on clones from round 12 and round 15,both of which had used PC-3^{luc} low invasive cells as the negative selection. The results showed that the SELEX procedure was yielding sequences that were more highly represented than would be expected by chance alone.

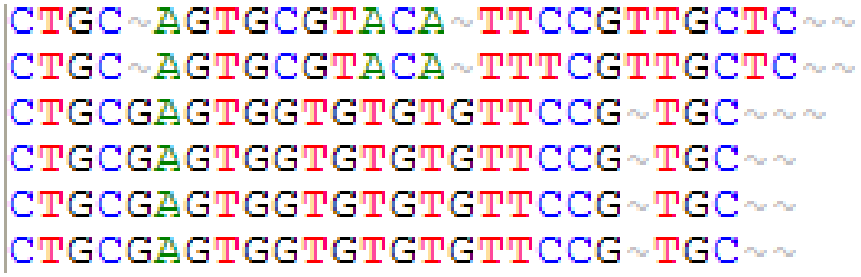
Figure 6, Sequencing Results. A) round 12, B) Round 15. Primer sequences omitted for simplicity. After twelve rounds, three of the sequences returned were present in duplicate. After fifteen rounds, only one sequence was present more than once, in four out of the thirteen clones returned, but this was also present in the round twelve sequence results. There were two other sequences that differed in just one nucleotide from each other and also showed considerable nucleotide sequence alignment with the highly represented sequences.

TABLE 1. Sequences from SELEX procedure.

A	Sequence	Length
1	TGTGGTGCGTTGGTGCATTTGTTTGCCTGTGGTTTGCTTGTTCGC	45
2	TGTGCGTGCTGTTTGCGTCTGTTGCTTTTTGTTGGTAATGTGGTG	45
3	TCTTTGTTCCTTGTCTGCTTTTTTGTGAGTGGTCTTGGTCGTGTG	45
4	TGCGTTGTGTTCTTCTTGTTTCATGTTATTCGTGTCCTCCGTTGT	45
5	TGTTGCGTAGCGTTGCTTCCTTGCATGTTGTGGTGTGTGTGTTG	44
6	CCACTGCGGATCCCATTCCCGTGCTTTTGC	30
7	CTGCGAGTGGTGTGTGTTCCGTGC	24
7	CTGCGAGTGGTGTGTGTTCCGTGC	24
8	TGAGGTTGCCGTTGCTTTGTG	21
9	CCTCGTTGGCGTGCGGTGCTT	21
9	CCTCGTTGGCGTGCGGTGCTT	21
10	TGTTGGTGTTGTTTTGGCT	19
11	GCCCACATCGCTCCTACA	18
11	GCCCACATCGCTCCTACA	18
B	**Sequence**	**Length**
1	TTGGTGCTTGCCTGTCCCTTCTACGTTTGTGCGAATGCC	39
2	TGCTATTGGTGGTCTTGTGTGGTGTTTTGGTGTG	34
3	TGCGTTGTACGGTACTTCTTTCCACGTGCGTTCC	34
4	TGTTGCTGGGATTAGCGCGTCTTCGTGGTTGTG	33

5	TGTTGCGCTGGTCAGTTTTGTCGTGTGTGTA	31
6	TGCACGATGCTTGGTTGTTTGTTGATCGG	29
7	TGCGCTGGATGTGTATGTTTGGCTG	25
8	CTGCAGTGCGTACATT***C***CGTTGCTC	25
8	CTGCAGTGCGTACATT***T***CGTTGCTC	25
9	CTGCGAGTGGTGTGTGTTCCGTGC	24
9	CTGCGAGTGGTGTGTGTTCCGTGC	24
9	CTGCGAGTGGTGTGTGTTCCGTGC	24
9	CTGCGAGTGGTGTGTGTTCCGTGC	24

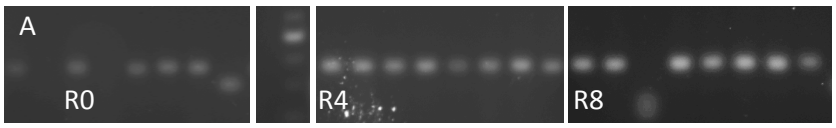
Figure 7, Sequence Alignment of Round 15 Sequences

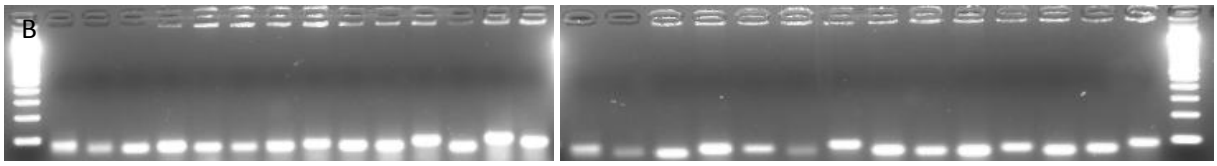


At this stage it was decided to use the highly represented sequence for further analysis, but also to continue with further rounds of SELEX.

An unexpected feature of the sequence results was that the though the library had a 45 base pair random sequence, after twelve rounds, only four of the fourteen returned sequences were full length and two were as short as eighteen. After fifteen rounds, all the returned sequences were between twenty four and thirty nine bases long. To determine the extent of the variability in sequence length, SELEX DNA from the library, round 4 and round 8 was cloned and colony PCR performed. R12 and R15 had already been shown to have fragments of different sizes, but a selection of R15 clones were amplified to give a better idea of the range of sizes

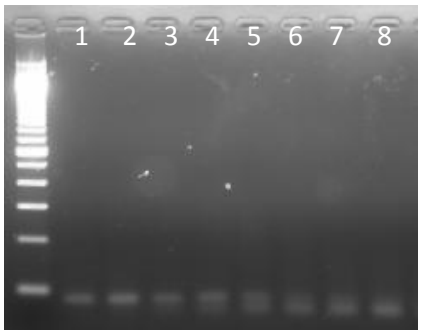
Figure 8, PCR of clones from different rounds of SELEX. SELEX DNA from the library, round4, round8 and round 15 was amplified by PCR using non biotinylated primers and cloned into PCR2.1 TOPO. Colony PCR was then performed using the same primers and the result run on a gel. A) R0, 4 and 8. One clone from the amplified library showed a smaller size. All the round 4 clones and R8 clones that successfully amplified were the same size as the library PCR, 85bp. B) R15, twenty eight clones were selected and amplified showing a wide range of product sizes





PCR of the pool of DNA for each round showed what was apparently a single band and a discrete product. To determine if there was a PCR bias, a mixture of library and R18 amplified together. No evidence of preferential amplification of shorter sequence was detected.

Figure 9, PCR of mixtures of library and Round 18 product. ssDNA library and R18 product at equal concentrations were mixed at different ratios and amplified. Lane 4, with 6µl library (full size) and 4µl R18 and lane 5 (4µl Library and 6µl R18) show similar amplification.



1) 1µl library
2) 9µl library + 1µl R18
3) 8µl library + 2µl R18
4) 6µl library + 4µl R18
5) 4µl library + 6µl R18
6) 2µl library + 8µl R18
7) 1µl library + 9µl R18
8) 10µl R18

KEY RESEARCH ACCOMPLISHMENTS

- Determined that although the initial RNA aptamers were effective, the effect was not robust.
- Developed DNA SELEX method
- Accomplished 18 rounds of SELEX
- Identified several aptamers that had increased representation from the SELEX procedure

REPORTABLE OUTCOMES

None

CONCLUSION

We have are developing DNA-based anti-invasive aptamers through development and use of a SELEX scheme that allows a full round of SELEX over the period of two days. We have demonstrated that specific sequences have been enriched, indicating the method is successful. We are continuing through several more rounds of SELEX then will use a new sequencing strategy to sequence a proportion of the entire pool of SELEX output aptamers to identify highly represented sequences. This strategy will be a marked improvement over cloning aptamers and selecting a very limited number of clones for sequencing. This will allow us to rapidly identify 1000s of aptamers and select those that are highly

represented. We will then quickly identify a set of candidates and identify which show enhanced and specific binding. We would then be able to select the best candidates and determine which have an inhibitory effect on cells and in vivo models.